CLEAN VERSION

IN THE CLAIMS

Please delete claims 1-25 and 35-58 without prejudice to presentation of claims of similar or identical scope in this or a later-filed case.

Please amend the following claims as indicated:

26. (AMENDED) A method of inhibiting viral infection comprising, introducing into cells a Phospholipid Scramblase polypeptide or fragments thereof, wherein the Phospholipid Scramblase polypeptide or fragment thereof contains the amino acid sequence PPxY and prevents virus budding.

29. (AMENDED) The method of claim 26, wherein the viral infection functions by being enveloped by a membrane envelope derived from the cell.

30. (AMENDED) The method of claim 26, wherein the Phospholipid Scramblase polypeptides and fragments thereof bind to proteins containing one or more WW domain sequence motifs.

34. (AMENDED) The method of claim 26, wherein the fragments are peptidomimetics of the Phospholipid Scramblase polypeptides.

Please add new claims 59-67:

59. (NEW) A method of preventing viral infection comprising, introducing a Phospholipid Scramblase polypeptide or fragments thereof into a cell, wherein said Phospholipid Scramblase polypeptide or fragments thereof contains the amino acid sequence PPxY and prevents virus budding.

60. (NEW) The method of claim 59, wherein the viral infection is an infection of a virus selected from the group consisting of a rhabdovirus, a filovirus, a retrovirus, a flavivirus, a coronavirus, a orthomyxovirus, a hubyavirus, a hepadnavirus, a herpesvirus, a poxvirus, a togavirus, a iridovirus, a paramyxovirus and a arenavirus.

61. (NEW) The method of claim 60, wherein the viral infection is selected from the group consisting of an HIV infection, an Ebola virus infection, a Marburg virus infection, and a Rabies virus infection.

62. (NEW) The method of claim 59, wherein the viral infection functions by being enveloped by a membrane envelope derived from the cell.

63. (NEW) The method of claim 59, wherein the Phospholipid Scramblase polypeptides and fragments bind to proteins containing one or more WW domain sequence motifs.

64. (NEW) The method of claim 59, wherein the Phospholipid Scramblase polypeptide is interferon-inducible.

65. (NEW) The method of claim 64, wherein the Phosopholipid Scramblase polypepetide has the amino acid sequence as set forth in SEQ ID NO:1 and SEQ ID NO: 2.

66. (NEW) The method of claim 59, further comprising administering an interferon.

67. (NEW) The method of claim 59, wherein the fragments are peptidomimetics of the Phospholipid Scramblase polypeptides.

IN THE SPECIFICATION

Please amend the specification as indicated:

Paragraph [0037] (AMENDED) Figure 11 shows gene structure of the HuPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) gene. Top:Schematic showing location of intron-extron borders within the HuPLSCR1, (SEQ ID NO:1 and SEQ ID NO: 2), genomic sequence of approx. 28 kb. Exons are represented by vertical lines. *Below*: Location of exons within the cDNA are indicated by arrows. Genomic sequence for HuPLSCR1, (SEQ ID NO:1 and SEQ ID NO: 2), deposited under GenBank accession no. AF224492.

Paragraph [0046] (AMENDED) Despite over forty years of research on IFNs, there are substantial gaps in our understanding of the molecular mechanisms that are responsible for the biological effects of IFNs. For example, triply deficient mice lacking RNase L, PKR and Mx1 can still mount a very substantial residual anti-viral effect in response to IFN-α. We have recently identified phospholipid scramblase-1 (PLSCR1)- a plasma membrane protein- as a new member of the IFN-regulated gene family. The purpose of the proposed studies is to gain an understanding of the cellular and plasma membrane changes induced through IFN's

a6 cont'd transcriptional upregulation of PLSCR1, and how these changes mediated through PLSCR1 potentially relate to the biologic activity of IFN in vivo.

Paragraph [0050] (AMENDED) During the past three years, we have made considerable progress in defining potential molecular mechanisms underlying this Ca^{2+} -induced transbilayer movement of plasma membrane PL. We reported the purification and characterization of an integral erythrocyte membrane protein (designated "*PL scramblase*; **hu PLSCR1**" (SEQ ID NO: 1 and SEQ ID NO: 2)) that, when reconstituted in liposomes, mediates a Ca^{2+} -dependent and pH-dependent accelerated transbilayer movement of all PL, mimicking the reorganization of plasma membrane PL observed either upon elevation of $(Ca^{2+})_c$ or upon acidification of the cytosol. There is also evidence that the same protein mediates similar function in platelets. The properties of this protein in PL bilayer membranes indicate that PLSCR1 is responsible for accelerated transbilayer movement of PS and other plasma membrane PL in all cells and tissues exposed to elevated $(Ca^{2+})_c$, arising as a consequence of immune injury or agonist-induced cell activation, and potentially, during cell senescence (*see below*).

a7 cont'd.

Paragraph [0051] (AMENDED) The deduced sequence of human PLSCR1 reveals a proline-rich acidic protein (35.1 kD; pKa=4.85) with a single predicted transmembrane domain near the C-terminus. There is also a single potential protein kinase C phosphorylation site (Thr^{161}) and an apparent EF-hand related Ca^{+2}-binding motif (see below). Analysis of the cDNA-derived protein sequence predicts a strongly preferred inside-to-outside orientation of the predicted 19-residue transmembrane domain, consistent with a type 2 plasma membrane protein. Thus, the bulk of the protein is predicted to extend from the cytoplasmic membrane leaflet, leaving a short extracellular tail. The predicted orientation of this protein is consistent with the anticipated topology of PL scramblase in erythrocytes and platelets, where the lipid-mobilizing function is responsive to Ca^{2+}or to acidification (pH<6.5) only at the endofacial surface of the plasma membrane. Northern blotting revealed that PLSCR1 mRNA was present in a variety of hematological and non-hematological cells and tissues. We have identified the residues in PLSCR1 that function in binding Ca^{2+} and our data suggest that the activity of PLSCR1 is also regulated post-translationally through palmitoylation at one or more Cys thiols. We have recently identified three additional members of the PL scramblase gene family (hu PLSCR 2-4 (SEQ ID

NOS: 3, 4, 5, 6, 7 and 8)) and the putative PL scramblase orthologues in mouse (mu PLSCR1-4 (SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15 and 16)) have been identified.

Pagagraph [0052] (AMENDED) We have shown that PLSCR1 is induced in IFN treated cells where it localizes to the plasma membrane, the site of budding for these viruses. The N-terminal regions of hu and mu PLSCR1, (SEQ ID NO:1 and SEQ ID NO: 2 and SEQ ID NO: 9 and SEQ ID NO: 10), share with diverse types of membrane bound viruses late function PPxY motifs required for release of virus particles from cells. PPxY motifs in PLSCR1 suppress virus budding by competing with the viral M or Gag proteins for binding to cellular WW domain proteins. This is the first example of host mimicry of a viral protein for the purpose of suppressing virus infections.

Paragraph [0055] (AMENDED) A potential link between PLSCR1 gene expression and neoplastic cell transformation was recently suggested by Kasukabe and associates. They describe a gene transcript (designated **NOR1**) that is markedly down-regulated in transformed murine monocytic cell lines relative to its expression in normal blood monocytes, and, a 5'-truncated form of this same transcript (designated **TRA1**) expressed only in leukemogenic mouse monocytic cell lines (but not expressed in normal monocyte or non-leukemogenic monocytic cell lines). Butyrate induction of monocytes to macrophages was accompanied by induced expression of the NOR1 transcript. They suggest that the truncated TRA1 gene product is associated with leukemogenesis in vivo, whereas increased NOR1 expression is associated with macrophage differentiation. Analysis of the open reading frame predicted by the NOR1 and TRA1 cDNA sequences reveals near-identity of protein sequence with hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), in overlapping portions of each polypeptide. This suggests that NOR1 (expressed in normal mouse monocytes and other tissues) is the murine orthologue of human PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) (mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10)) whereas the TRA1 gene product, found only in leukemogenic cell lines, is a truncated form of mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) that arises through alternative splicing (deleting exons 1-5). Down-regulation of wild-type mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) (i.e. NOR1) in transformed monocytes and the de novo expression of the alternatively-spliced, truncated form of this protein (i.e., TRA1) in only leukemogenic subclones, suggests that NOR1 (and thus

presumably PLSCR1) is required for normal cell senescence, whereas mutant TRA1 might promote leukemogenic potential, potentially as a dominant-negative PL scramblase inhibitor.

Paragraph [0056] (AMENDED) As was noted, we now have shown that the expression of hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) is markedly upregulated by IFN. PLSCR1 directly contributes to the antiproliferative action of IFN and provides a causal explanation for the observed association of aberrant NOR1/TRA1 gene expression in transformed and leukemogenic cell lines.

Paragraph [0135] (AMENDED) Analysis of 5' flanking genomic sequence in reporter constructs showed that transcriptional control of PLSCR1 was entirely regulated by a single IFN-stimulated response element (ISRE) located in the first exon. A similar induction of PLSCR1 by IFN-α2a was also observed in a variety of other human tumor cell lines as well as in human umbilical vein endothelial cells. In these cell lines, the marked increase in PLSCR1 expression by IFN-α2a was not accompanied by increased cell surface exposure of PS. These results suggest that remodeling of the cell surface requires both exposure to IFN and a second yet-to-be identified event to stimulate plasma membrane PL scramblase activity and to mobilize PS to the cell surface. We have recently confirmed that the IFN-inducibility observed for hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) is also shared by the murine orthologue, mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10). Experiments to identify the ISRE(s) responsible for IFN-regulated expression of mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) gene (as described for hu PLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2)) are now in progress.

Paragraph [0136] (AMENDED) To directly measure the effect of PLSCR1 induction on tumor growth in the absence of other IFN induced proteins, we constitutively expressed hu PLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) cDNA in the human ovarian cancer cell line, HEY1B. The hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA was subcloned under the control of a CMV promoter in plasmid vector, pcDNAneo3. Transfection of the HEY1B cells was followed by selection in media containing G418. Western blots probed with a monoclonal antibody to PLSCR1 (mab 4D2) revealed that only one of fifty clones expressed high levels of PLSCR1. The clone, S48, expressed about 4-fold more PLSCR1 than the parental cells. This

compares with the 10-fold increase in PLSCR1 levels obtained with IFN-α treatment of the cells. The in vitro growth rates of the S48 cells and of the clonal cell line (V24) containing the empty vector was determined in the presence and absence of 1,000 units per ml of IFN-α2a . While the growth of both cell lines was modestly suppressed by IFN, there was no difference in the growth rates of the V24 and S48 cells. In sharp contrast, there was a dramatic difference in the ability of the two cell lines to form tumors after being implanted into nude mice. In these experiments, the V24 cells and the PLSCR1 expressing clone, S48, (10^6 cells/site) were injected subcutaneously (s.c.) into the flanks of groups of six nude mice. Tumor growth was monitored every 3-4 days with a caliper and the excised tumors were measured upon termination of the experiment. The tumor growth rate of the empty-vector control clone was about 8-fold higher than that of the PLSCR1 (S48) clone. To rule out clonal variation as the cause of the differences, we cloned hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA into vector pIREShyg (Clontech) which expresses a bicistronic mRNA under the control of a CMV promoter. The first open reading frame is PLSCR1 followed by an internal ribosome entry site (IRES) and a hygromycin B phosphotransferase sequence. Therefore, after transfection of the HEY1B cells and selection in hygromycin-containing media, expression of PLSCR1 was tightly coupled to hygromycin resistance. The result was high expression (about 4- to 10-fold over basal levels) of PLSCR1 in every clone analyzed and as well as in the pool of selected cells (data not shown). The pools of PLSCR1 expressing cells and the empty-vector pool of cells were inoculated s.c. into the flanks of groups of nude mice. The tumor results obtained from the uncloned pooled cells (transfected with PLSCR1 cDNA) were similar to those obtained for the S48 clone, thus eliminating a clonal artefact as the basis for the anti-tumor activity of PLSCR1 (data not shown). In this proposal we will investigate the molecular and cellular mechanism of the anti-tumor effect of PLSCR1.

Paragraph [0137] (AMENDED) To determine the possible antiviral function of PLSCR1, cell viability and viral yield assays with VSV were performed in the presence or absence of IFN-β on cells expressing hu PLSCR1, (SEQ ID NO:1 and SEQ ID NO: 2), cDNA. VSV is a rhabdovirus containing a negative RNA genome with five genes in the order 3'-N-P-M-G-L-5'. Its virions are composed of two main parts, a nucleocapsid or ribonucleoprotein (RNP) core and a lipid bilayer envelope. VSV was chosen for these studies because IFN inhibits its replication at a late stage and it is a membrane-bound virus with a PPxY viral budding motif in

its M protein. Hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) is an IFN-induced membrane protein that contains two PPxY motifs near its cytoplasmic N-terminus. Therefore, PLSCR1 could interfere with VSV budding or assembly by competing with VSV M protein for binding to cellular WW domain proteins required for these late viral processes.

Paragraph [0140] (AMENDED) BLAST analysis of the GenBank EST database using hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA revealed three different families of EST clones that were similar, but distinctly different from the sequence we originally reported for hu PL scramblase (PLSCR1), (SEQ ID NO:1 and SEQ ID NO: 2). In order to obtain cDNAs for these putative homologues of huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), the relevant EST clones were used to design PCR primers. Full length cDNAs were obtained by PCR using cDNA from multiple human tissues as templates. As illustrated, the cloned cDNAs encode three novel proteins with high homology to huPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) (FIG. 9). The predicted open reading frames of these putative homologues show sequence identities to huPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) of 59% (huPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4); 224 AA; GenBank AF159441), 47% (huPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6); 295 AA; GenBank AF159442) and 46% (huPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8); 329 AA; GenBank AF199023), respectively. Corresponding cDNAs of putative murine orthologues of each of the four hu PLSCR (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8) family members have also been identified, and similar proteins of unknown function are also predicted in the C. elegans, Drosophila, and porcine genome (data not shown).

Paragraph [0141] (AMENDED) Inspection of the four human PLSCR (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8) homologues reveals a low degree of similarity for the proline-rich N-terminal portion of the proteins (AA 1 to 85 in huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2)), and highest degree of identity towards the C-terminus. This includes a highly conserved segment (AA 273 to 284 in huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2)) which has been shown in huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) to contain the Ca^{2+}-binding site. The sequence of huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) predicts a type II membrane protein with single transmembrane domain near the C-terminus (AA 291-309), and most of the polypeptide (AA 1-290) extending into the cytosol. By contrast, such predictions are ambiguous for the

newly described homologues, as are predictions of putative intracellular localization. Of note, the predicted open reading frame for huPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4), the closest homologue to huPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), is missing the proline-rich N-terminus that is characteristic for all other members of this family. As was discussed, this segment in PLSCR1 that is missing in PLSCR2 contains PPxY and PxxP motifs which may serve as binding sites for proteins containing WW or SH3 domains, respectively, and potentially confer on PLSCR1 anti-viral activity. The PPxY and PxxP motifs common to hu PLSCR1, PLSCR3, & PLSCR4 (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8) are also conserved in the corresponding mouse orthologue (FIG. 10) as well as in the potential porcine (GENBank F14810) and C. elegans (GENBank Z82084,AF078785) orthologues of PLSCR1. Although binding partners for any of the PLSCR proteins have not been identified to date, it is interesting to note that the functional implication of the missing N-terminal segment in huPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) may also be a potential loss of interaction with an adaptor or signaling molecule. A similar truncation deleting the proline-rich segment of mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) has previously been associated with leukemogenesis.

Paragraph [0142] (AMENDED) ***Chromosomal assignment of PL scramblase family members***. Chromosomal localization by analysis of STS sequences of the NCBI Human Gene Map'99 and/or radiation panel hybrid mapping revealed that the genes for hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), hu PLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4), and PLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) are tightly clustered between markers D3S1557 and D3S1306 (164.6-168.3 cM) on chromosome 3 (3q23) at the physical location 537.09 cR_{3000} (P130). HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) was also mapped to chromosome 3 at 3q23 by fluorescence in situ hybridization. By contrast, PLSCR3 maps to chromosome 17 (p13.1) between markers D17S1828 and D17S786 (9.8-18.1 cM) at the physical location 53.50 cR_{3000} (P0.90).

Paragraph [0143] (AMENDED) ***Tissue Distribution of PL Scramblase Family Members***. Initial insight into the tissue distribution of the four newly-identified members of the PLSCR gene family was obtained by Northern blotting with ^{32}P-labeled probes specific for huPLSCR1 to huPLSCR4 (SEQ ID NOS: 1-8), respectively. Transcripts for PLSCR1 (~2400 bp

and ~1600 bp) were expressed in spleen, thymus, prostate, testis, uterus, small intestine, colon, peripheral blood lymphocytes, heart, placenta, lung, liver, kidney and pancreas, but below the limits of detection in brain and skeletal muscle. By contrast, PLSCR2 (~1600 bp) was detected only in testis. PLSCR3 (~2400 bp and ~1600 bp) was detected in spleen, thymus, prostate, uterus, small intestine, colon, PBL, skeletal muscle, heart, placenta, lung, kidney and pancreas, but not in testis, brain or liver. PLSCR4 (~3600 bp) was detected in all tissues examined except peripheral blood lymphocytes, and was the only PLSCR family member detected in brain.

Paragraph [0144] (AMENDED) ***Antibody Probes of PLSCR Family Members***. We have several monoclonal antibodies specific for hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) that do not cross-react with hu PLSCR2-4 (SEQ ID NOS: 3, 4, 5, 6, 7 and 8). Two of the mabs have been found to cross-react with mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) and can be used to selectively monitor expression of the protein in murine cells and tissue. In order to develop additional antibody probes, peptides corresponding to unique sequence identified in the various PLSCR homologues have been synthesized and peptide-KLH conjugates injected into rabbits for antisera production.

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Paragraph [0145] (AMENDED) These antisera were analyzed by ELISA and Western blotting against recombinant PLSCR1-4 produced in E. coli as MBP-fusion proteins. At the present time, we have available high titer antisera selective for hu and mu PLSCR1-3 (SEQ ID NOS: 1, 2, 3, 4, 5 and 6) and (SEQ ID NOS: 9, 10, 11, 12, 13 and 14). Immunizations with peptides derived from hu and mu PLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8 and SEQ ID NO: 15 and SEQ ID NO: 16) are now in progress. Thus, we anticipate that antibody reagents suitable for monitoring selective protein expression of each of the four PLSCR family members in both human and mouse cells and tissues will be available.

Paragraph [0146] (AMENDED) Under contract between Scripps/BCSEW and Lexicon Genetics, Inc., we initiated genomic cloning and Cre-Lox targeted disruption of the murine PLSCR gene locus in 1998. This was prior to our discovery that PLSCR is a multigene family of proteins in both mouse and man, currently shown to include four expressed genes. We now recognize that the original mouse orthologue of human PL scramblase that we had cloned and

targeted for gene disruption is mu PLSCR2 (SEQ ID NO: 11 and SEQ ID NO: 12), not the true orthologue of hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2). We therefore recently cloned and targeted disruption of the mu PLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) gene (**see FIG. 10**). In both cases (PLSCR1 & PLSCR2), the targeting construct was designed to disrupt exon 8, which (by sequence alignment to hu PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2)) is predicted to contain the Ca^{2+} binding and putative transmembrane segments of the proteins. The PLSCR2 knockout was completed last year and the breeding colonies transferred to Scripps for use in this Project. To date, no abnormality has been identified in the homozygote $PLSCR2^{-/-}$ animals. As of date of submission of this application (01/29/00) matings of the chimeric mice containing the PLSCR1 gene disruption were performed, and 14 resulting agouti pups are now being analyzed for germline transmission. No information is now available on the viability or phenotype of the homozygous $PLSCR1^{-/-}$ animals.

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Paragraph [0147] (AMENDED) **Cloning of Human PL Scramblase 1 Gene**. A BAC-human genomic library (Genome System Inc., St. Louis) was screened with a 1.445 kb HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA probe (GenBank accession number AF098642) by hybridization. A positive clone of approximately 100 kb was obtained, digested with EcoRI, and the fragments were cloned into pcDNA3 (Invitrogen). Subclones were identified by hybridization with digoxigenin-labeled HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA probe, and DNA inserts were sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

Paragraph [0148] (AMENDED) **Construction of 5' Flanking Region Deletions of PLSCR1 Gene.** A 4180 bp DNA fragment consisting of the 5'flanking region (0 to -4120) and the first 60 bp of the first exon (+1 to +60) of the HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) gene was cloned into pGL3-basic-luciferase reporter vector (Promega). In order to identify the promoter region of the gene, the 5' flanking DNA was serially deleted both from the 5' and the 3' end by PCR-mediated deletion and cloned into pGL3-basic-luciferase reporter vector for analysis.

Paragraph [0149] (AMENDED) **Cell Culture and Transfection of Daudi Cells**. The Burkitt's B cell lymphoma cell line Daudi was cultured in RPMI 1640 complete medium with 20% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml, at 37 °C., 5% CO_2 Cells were washed and suspended to 1.35 x 10^7/ml in OPTI-MEM. To 0.8 ml of cell suspension in a 0.4 cm electroporation cuvette, 20 μg of pGL3-5'flanking region or deletions of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) and 20 μg of pSV-β-galactosidase (Promega) were added. The mixture was incubated for 10 min on ice, and electroporated at 380 V and 500 μF using a Bio-Rad Gene Pulser II (Bio-Rad). After incubation for 10 min at 37. °C. the cells were plated in 10 ml of RPMI complete medium. Forty-eight hours later, transfected cells were harvested for luciferase and β-galactosidase assay.

Paragraph [0151] (AMENDED) **Cloning of PL Scramblase Family Members**. Blast search of the GenBank database of expressed sequence tags (EST) with HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) cDNA identified three distinct clusters of EST clones each displaying overlapping identities. Appropriate EST clones were obtained from American Type Culture Collection, sequenced, and the information was used to design PCR primers specific for the 3' and 5' ends of the various homologues. Full length cDNAs were obtained by PCR using a human erythroleukemia cell (HEL) cDNA library (Clontech; for HuPLSCR2 and HuPLSCR3 (SEQ ID NOS: 3, 4, 5 and 6)) or human multiple tissue cDNA (Clontech; pancreas; for HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8)) as template. Each PCR reaction and cloning was performed in triplicates, and Advantage HP_2 DNA polymerase mix (Clontech) was used to decrease PCR-mediated error. PCR products were cloned into pCR2.1 (Invitrogen) for sequencing.

Paragraph [0152] (AMENDED) **Chromosomal mapping.** The chromosomal location for HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) was determined using the GeneBridge 3 Human Radiation hybrid panel and oligonucleotides 5'-CCTGGTGCTTAGGGTAGACAATATG-3' and 5'-CTGACGTCCTGGGTAGAAGGCCTGGG-3' as the forward and reverse primers, respectively (Research Genetics, Huntsville, AL). The primers flank a small intron (88bp) within the 5' untranslated region of HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4), giving a PCR product of

314 base pairs. The map position was calculated using the Stanford server (on the world wide web at URL address:-shgc.Stanford.edu).

Paragraph [0153] (AMENDED) **Tissue Distribution**. Human multi-tissue Northern blots (Clontech) were hybridized to random prime labeled cDNA probes of each HuPLSCR (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8) family member. The HuPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) probe consisted of the 5' 498 bp of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) (gbAF098642). The HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) probe (1265 bp) was prepared by digesting EST clone AA813518 with Not1 and Xho1, and the HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) probe (851 bp) by digesting EST clone N78598 with Not1 and Xho1. The cDNA fragments were separated from vector sequences by agarose gel electrophoresis and purifed using Wizard columns (Promega). The cDNA probes were labeled with α-^{32}P-dATP (50 μCi/25 ng cDNA, 3000 Ci/mmol; ICN) using the random prime labeling kit from Boehringer Mannheim to a specific activity $\geq 1 \times 10^9$ dpm/μg. Due to non-specific hybridization of the cDNA probe, an RNA antisense probe was designed for HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6). A PCR product of the 3' untranslated region of HuPLSCR3, (SEQ ID NO: 5 and SEQ ID NO: 6), was prepared using the forward primer 5'-TGTGAGGAGACCATCACCTCGAC-3' and reverse primer 5'-AAAGCTGATATGCCTGTGTGCC-3'. The reverse primer contained the T7 promoter sequence (5'-AATTTAATACGACTCACTATAGGG-3') at the 5' end. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen). A ^{32}P-labeled antisense RNA probe was prepared using 50 ng of the PCR product as template in T7 transcription reaction with α-^{32}P-UTP (800 Ci/mmol; 20 μCi/.mu.l: Amersham) following the instructions included in the T7 Strip-EZ RNA kit (Ambion). Multi-tissue Northern blots were prehybridized for 1 hour at 68 °C. in ExpressHyb hybridization buffer (Clontech) followed by hybridization for 18 hours at 68 °C. in the same buffer containing 2×10^6 cpm/ml denatured random prime-labeled probe. For HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6), the blots were prehybridized in Ultrahyb hybridization buffer (Ambion) with 100 μg/ml denatured salmon sperm DNA and 50 μg/ml yeast RNA and hybridized in the same buffer containing ^{32}P-labeled antisense RNA probe (2×10^6 cpm/ml) at 68 °C. for 18 hours. The blots were washed at a final stringency of 0.1.times. SSC in 0.1% SDS at 50 °C. (68 °C. for HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6)), and exposed to Amersham Hyperfilm MP.

Paragraph [0154] (AMENDED) **Human PLSCR1 Gene Structure.** In order to gain insight into the gene organization of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), a clone of approximately 100 kb of genomic DNA was obtained from a BAC-human genomic library by screening with a HuPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2) Cdna probe. EcoR1 digested fragments were cloned into pcDNA3, and sequence from six different clones was used to deduce approximately 30 kb of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) genomic DNA. The organization of the gene was deduced by alignment of the genomic sequence with Cdna sequence for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) (GenBank accession number AF098642). The HuPLSCR1 (SEQ ID NO: 1 SEQ ID NO: 2) gene consists of 9 exons, 8 introns and 5' flanking sequence (deposited under GenBank accession numbers AF153715 and AF224492). As shown in Table 1, invariant gt and ag were found at the intron splice donor and acceptor sites. As illustrated in FIG. 1, the first exon is untranslated, with the open reading frame starting in exon 2. Of interest, Kasukabe et al. reported the occurrence of a truncated form of MuPLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) (termed MmTRA1a), the closest murine orthologue of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), in a mouse monocytic cell line which was highly leukemogenic when injected into syngeneic or athymic mice. In addition, non-leukemogenic sublines became leukemogenic when transfected with MmTRA1a. By contrast, normal macrophages expressed only full length MuPLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10). Comparison of the sequence of MmTRA1a with FIG. 1 reveals that murine MmTRA1a is likely a product of alternative splicing, as the predicted open reading frame reported by Kasukabe et al. starts at a position corresponding to the beginning of Exon 6 in HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2). It remains to be determined whether the analogous alternatively spliced forms of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) are similarly associated with leukemias in man.

Paragraph [0155] (AMENDED) **Promoter Analysis**. In order to identify the promoter region for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), luciferase reporter constructs of 5' flanking sequence and serial 5' or 3' deletions were expressed in Daudi cells. As illustrated by the data in FIGS. 2 and 3, a reporter construct containing 5' untranslated sequence comprised of -4120 to +60 exhibited strong promoter activity. Deletion of sequence from the 5' end from -4120

bp to -557 bp did not affect promoter activity (FIG. 2). However, deletion from -95 bp of 5' flanking sequence to +60 bp of the first (untranslated) exon resulted in the loss of more than 97% of promoter activity, locating the promoter of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) to that region (FIG. 3). Computer analysis of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) 5' flanking sequence using the MatInspector V2.2 program (on the world wide web at URL address: gsf.de/biodv/matinspector.html) revealed two GC boxes (TAGGGGAGGGGCCT at -79 bp to -66 bp, and AGGAGGTGGGCGCA at -59 bp to -46 bp) and a CCAAT box (TCTCTCCAATG at -111 bp to -101 bp) (FIG. 4), consistent with the data in FIG. 3 locating promoter activity to that region. In addition, potential binding sites for transcriptional activators, including activator protein 4 (AP4, upstream stimulating factor (USF), eurkaryotic transcriptional regulator 1 (ETS1), interferon-stimulated response element (ISRE), and interferon regulatory factor (IRF), were identified . We had previously identified the single ISRE that is located in the first untranslated exon (+21 to +35) as the primary site responsible for the upregulation of HuPLSCR1, (SEQ ID NO:1 and SEQ ID NO: 2), by interferon-α

Paragraph [0156] (AMENDED) **Identity of a Novel PL Scramblase Gene Family.** Upon performing BLAST searches of the GenBank EST database with human PL scramblase 1 (HuPLSCR1) (SEQ ID NO: 1 and SEQ ID NO: 2), we noted three distinct clusters of EST clones that were similar, but distinctly different from the sequence we had originally reported for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2). In order to obtain cDNAs for these putative homologues of HuPLSCR1 (SEQ ID NO:1 and SEQ ID NO: 2), sequence derived from relevant EST clones was used to design PCR primers. Full length cDNAs were obtained by PCR using a cDNA library from human erythroleukemia cells (HEL), and cDNA from multiple human tissues as template. As illustrated in FIG. 5, the cloned cDNAs encode three novel proteins with high homology to HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2). The predicted open reading frames encode proteins with 59% (HuPLSCR2 (SEQ ID NO:3 and SEQ ID NO: 4); 224 AA; GenBank AF159441), 47% (HuPLSCR3 (SEQ ID NO:5 and SEQ ID NO: 6); 295 AA; GenBank AF159442) and 46% (HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8); 329 AA; GenBank AF199023) identity, respectively, to HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2). Furthermore, cDNAs of novel murine orthologues of HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6) (MuPLSCR3 (SEQ ID NO: 13 and SEQ ID NO: 14); 327 AA; GenBank AF159850) and

HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) (MuPLSCR4 (SEQ ID NO: 15 and SEQ ID NO: 16); partial, in progress) have been cloned and sequenced (data not shown). Closer inspection of the four human PLSCR (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8) homologues reveals low degree of similarity for the proline-rich aminoterminal portion of the proteins (amino acids 1 to 85 in HuPLSCR1) (SEQ ID NO: 1 and SEQ ID NO: 2), and highest degree of identity towards the carboxyterminus, including a region (AA 273 to 284 in HuPLSCR1, SEQ ID NO: 1 and SEQ ID NO: 2) which has been shown for huPLSCR1, (SEQ ID NO: 1 and SEQ ID NO: 2), to contain a Ca^{2+}-binding site required for the Ca^{2+}-induced transmembrane movement of phospholipids. We have previously noted that computer analysis of HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) predicts a type II protein with a transmembrane domain near the carboxyterminus (AA 291-309), and most of the polypeptide (AA 1-290) extending into the cytosol. By contrast, such predictions are ambiguous for the newly described homologues, as are predictions of putative intracellular localization. Of note, the predicted open reading frame for HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4), the closest homologue to HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2), is missing the proline-rich aminoterminus that is characteristic for all other members of this family. As pointed out previously, this region also contains a number of PXXP motifs which may serve as potential binding sites for proteins containing SH3 domains. In addition, HuPLSCR1, 3, and 4 (SEQ ID NOS: 1, 2, 5, 6, 7 and 8) all contain one or more PPxY motifs, suggesting a potential interaction with proteins containing WW domains. Such domains are primarily found in proteins with signaling or regulatory function. Although binding partners for any of the PLSCR proteins have not been identified to date, it is interesting to note that the functional implication of the missing aminoterminal segment in HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) may be a potential loss of interaction with an adaptor or signaling molecule. A similar truncation has previously been noted to confer leukemogenic potential to MuPLSCR1 (SEQ ID NO: 9 and SEQ ID NO: 10) (MmTRA1a).

Paragraph [0157] (AMENDED) **Chromosomal assignment of PL scramblase family members**. The chromosomal locations of HuPLSCR1, HuPLSCR3 and HuPLSCR4 (SEQ ID NOS: 1, 2, 5, 6, 7 and 8) were determined from nucleotide sequence homologies to STS sequences found on the NCBI Human Gene Map '99 (on the world wide web at the URL address: ncbi.nlm.nih.gov/genemap/). The genes for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID

NO: 2) (stSG10277) and HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) (gb N78598/G37067) are clustered between markers D3S1557 and D3S1306 (164.6-168.3 Cm) on chromosome 3 (3q23) at the physical position 537.09 Cr_{3000}. HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) has also been independently mapped to chromosome 3 at 3q23 by fluorescence in situ hybridization. A partial sequence for the HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6) gene is located between nucleotide 10501 and 9174 of the gene sequence deposited in GenBank under gb AF097738, which also codes for a non-receptor tyrosine kinase gene (nucleotides 531-9180). The non-receptor tyrosine kinase gene has been localized to chromosome 17p13.1 between markers D17S1828 and D17S786 (9.8-18.1 Cm) at the physical position 53.50 Cr_{3000}, thus localizing HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6) to that position. The gene for HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) was mapped as described in *Experimental Procedures*, and was found to be located on chromosome 3 (3q23), closely clustered with HuPLSCR1(SEQ ID NO: 1 and SEQ ID NO: 2) and HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8), suggesting that these three homologues arose by gene duplication.

Amended. Paragraph [0158] (Amended) **Tissue Distribution of PL Scramblase Family Members.** The tissue distribution for the four members of the PL scramblase family of proteins was evaluated by Northern blotting with ^{32}P-labeled probes specific for HuPLSCR1 to HuPLSCR4 (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8), respectively. The specificity of the probes was ascertained by DNA dot blot (FIG. 6). Whereas amounts of mRNA for HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) in many of these tissues were below the limit of detection, the mRNA for the other three homologues were expressed in most of the 16 different tissues examined. However, the expression patterns for these three family members show distinct differences. FIG. 7 shows that mRNA for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) was below the limits of detection in brain and skeletal muscle. As previously reported, two different size transcripts (~2.55 kb and 1.6 kb) were detected for HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) in all tissues expressing this gene. Kasukabe et al. have suggested that the different size transcripts arise from alternative polyadenylation signals within the 3' untranslated region of the HuPLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) gene. Interestingly, the expression of HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) mRNA appears to be highly restricted. Although trace amounts of HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) could be amplified

from HEL cells through several rounds of PCR for sequencing purposes (see Experimental Procedures), a 1.6 kb message was only detected in testis. This result was confirmed by probing a human Multi Tissue Expression Array (Clontech, Cat. #7775-1), which again yielded a positive blot against mRNA of testis only. This blot also revealed that in addition to the tissues listed in FIG. 7, HuPLSCR2 (SEQ ID NO: 3 and SEQ ID NO: 4) message was also not detected in any tissues of the gastrointestinal tract, bladder, ovary, lymph node, bone marrow, and adrenal, thyroid, salivary or mammary gland (results not shown). HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6) mRNA was below the limit of detection in testis, brain or liver. Two sizes of mRNA were detected with the HuPLSCR3 (SEQ ID NO: 5 and SEQ ID NO: 6) specific probe: whereas a 1.8 kb mRNA species was observed for most tissues, a .about.2.1 kb mRNA transcript was detected in skeletal muscle. An mRNA transcript of 4 kb was detected for HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) in all tissues examined except peripheral blood lymphocytes. Importantly, HuPLSCR4 (SEQ ID NO: 7 and SEQ ID NO: 8) mRNA was the only family member expressed at detectable levels in brain tissue. Whether HuPLSCR1, 3, and 4 (SEQ ID NOS: 1, 2, 5, 6, 7 and 8) have redundant function in a number of tissues, or whether these proteins exhibit activities that are distinct for each family member is the subject of future experimentation.

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Page 56, Line 1 (AMENDED)

a12 **Table 1** Hu-PLSCR1, (SEQ ID NO: 1 and SEQ ID NO: 2), exon and intron sequences.

Paragraph [0165] (AMENDED) **Molecular Cloning of 5' Flanking Region of PLSCR1 Gene and Construction of Deletions.** Human PLSCR1 (SEQ ID NO: 1 and SEQ ID NO: 2) gene was cloned from a BAC-human genomic library (Genome System Inc., St. Louis, Mo.) using full length PLSCR1 cDNA for hybridization, and 4.12 kb of 5'flanking region was sequenced (GenBank AF153715). 4.18 kb DNA consisting of the 5'flanking region (-1 to -4120) and the first 60 bp of the first exon of the gene (+1 to +60) was amplified by PCR using Advantage DNA polymerase mix (CLONTECH Laboratories, Inc., Palo Alto, Calif.), and PCR products were cloned into pGL3-basic-luciferase reporter vector (Promega, Madison. Wis.). Analysis of the 5' flanking region for the presence of putative binding sites for transcription factors was performed using MatInspector V2.2. The four putative binding sites for ISGF3 or

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IRFs (see FIG. 5) were deleted by PCR-mediated deletion. All DNA sequencing was performed on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, Calif.).

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